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The tissue form of chicken type VI collagen

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We have purified intact type VI collagen from chicken gizzard. The protein was found to consist of a 130 kDa, a 140 kDa and a 180–200 kDa subunit. The 130 kDa and 140 kDa subunits were obtained in equimolar amounts and identified as the α2 (VI) and the α1 (VI) chains, respectively. The third subunit was usually obtained in the form of 3–4 closely related polypeptides, which may represent different processing or modification products of the α3 (VI) chain.

Collagen; Extracellular matrix; 140 kDa glycoprotein; (Chicken gizzard)

1. INTRODUCTION

Type VI collagen is an abundant component in the stroma of most organs (review [1]). The protein consists of collagenous as well as globular domains. The structure of the entire molecule, however, is not yet known in detail, because it is insoluble under nondenaturing conditions and therefore difficult to isolate. For that reason most studies have focused on the collagenous domain, which can easily be purified from peptic digests of various tissues [1]. Such pepsin-extracted type VI collagen consists of equimolar amounts of three genetically different polypeptide chains: α1(VI)-pepsin, α2(VI)-pepsin and α3(VI)-pepsin [2]. Indirect immunological methods were used to identify the intact, non-pepsinized subunits. A collagenase-sensitive component of 140 kDa was found that reacted with antibodies raised against the pepsin-extracted type VI collagen [3–9]. Consequently several authors proposed that the intact molecule consists of three different polypeptide chains of 140 kDa which migrate on polyacrylamide gels as a single broad band [4,7–9]. In contrast to these studies, we identified polypeptides with molecular masses of approx. 200 kDa, which were immunologically related to type VI collagen [10,17]. In order to solve this discrepancy it is inevitable to purify intact type VI collagen and to separate its individual subunits. As this has proved especially difficult with the human or bovine protein, we turned to an avian model and report here on the separation of the chicken type VI collagen into subunits of 130, 140 and 180–200 kDa.

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Abbreviation: PAS reagent, periodate/Schiff reagent

2. EXPERIMENTAL

2.1. *Preparation of type VI collagen*

Chicken gizzard (100 g, wet wt) was homogenized and extracted successively with (i) 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, (ii) 1 M NaCl, 50 mM Tris-HCl, pH 7.5, (iii) 2% (w/v) SDS, 50 mM Tris-HCl, pH 7.5 essentially as described [10]. Each buffer contained a cocktail of protease inhibitors (1 mM *N*-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA). The SDS-extract was concentrated by ultrafiltration (Amicon; PM-10), dialyzed against 100 mM NaCl, 0.2% SDS, 1 mM EDTA, 50 mM Tris-HCl,

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pH 7.5, and denatured by heating at 80°C for 5 min. After centrifugation, the solubilized proteins were separated on a column of Sephacryl S-500 (Pharmacia, 3.3 × 70 cm, 17.4 ml/h) at room temperature [17]. Aliquots of the effluent were analyzed on polyacrylamide gels. Type VI collagen containing fractions were reduced with 2-mercaptoethanol, alkylated with 4-vinylpyridine [11] and chromatographed on Sephacryl S-400 as above (2.5 × 67 cm, 13.7 ml/h). In addition, pepsinized type VI collagen was prepared from the same tissue by the method of Odermatt et al. [12].

2.2. *Gel electrophoresis*

Proteins were analyzed on gradient polyacrylamide gels as described by Laemmli [13]. Polypeptides were stained with Coomassie brilliant blue or detected by silver staining [14]. Glycoproteins were visualized by the PAS reagent [15]. Gels were scanned with a CD 50 densitometer (DESAGA, Heidelberg).

2.3. *Peptide mapping*

Peptide mapping was performed according to Cleveland [16]. Subunits of type VI collagen were separated on a 5–10% polyacrylamide gel in the presence of 2-mercaptoethanol. Bands of interest were excised, placed into the sample wells of a 10–15% polyacrylamide gel and overlaid with a protease containing buffer. Electrophoresis was performed until the dye front reached the separating gel. Then, the current was turned off and digestion was allowed to proceed for 1 h. After completion of electrophoresis the peptides generated were detected by silver staining.

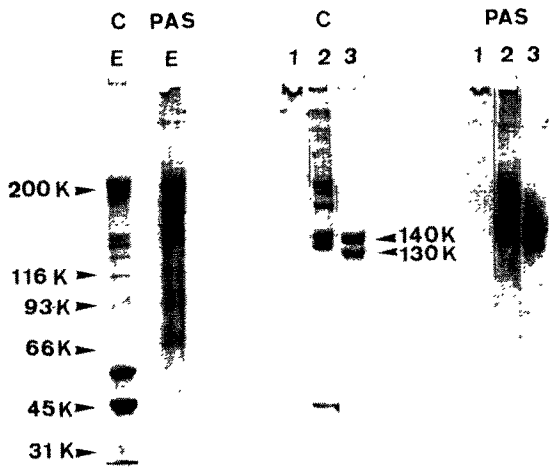

Fig.1. Purification of chicken type VI collagen. Polypeptides were resolved on 5–10% gradient gels under nonreducing (lanes 1) or reducing (lanes E, 2, 3) conditions and stained with Coomassie blue (C) or the PAS reagent (PAS). Lane E, crude SDS extract. Lanes 1 and 2, type VI collagen after purification on Sephacryl S-500. Lane 3, 130/140 kDa subunits after purification on Sephacryl S-400. The migration positions of globular protein standards are given in the left margin.

2.4. *Immunological procedures*

Antisera against chicken type VI collagen were raised in rabbits as described [10,17]. The sera were affinity purified on pepsin-extracted type VI collagen or on DEAE-purified 140/130 kDa subunits [3], that had been coupled to Affigel-10 (Biorad). Residual cross-reaction was eliminated by soaking little stripes of nitrocellulose to which the cross-reacting material was adsorbed in the antibody solution. The immunoblotting technique was performed as described [10,18].

3. RESULTS

An SDS extract obtained from chicken gizzard showed three major bands of 45, 55 and 210 kDa and various minor bands when analyzed on a polyacrylamide gel stained with Coomassie blue (fig.1). With the PAS reagent, only some minor bands of 130, 140 and 180–200 kDa gave a positive reaction. These minor polypeptides were susceptible to digestion with bacterial collagenase, provided that all SDS had previously been removed from the extract. The other bands visible on the Coomassie blue stained gel, however, were not affected by such a treatment. We presume that at least some of the collagenase-sensitive bands represent subunits of type VI collagen as this collagen is known to be heavily glycosylated [19].

In order to purify type VI collagen, the SDS extract was fractionated on a column of Sephacryl S-500. Four peaks were obtained as had been observed with a similar extract from bovine uterus [17]. Only the second peak contained proteinaceous material that changed its migration behaviour on a polyacrylamide gel by the addition of 2-mercaptoethanol and therefore only this peak was further investigated. The proteinaceous material consisted of disulfide-bonded aggregates

which did not enter a 5% stacking gel, but split into polypeptides of 130, 140 and 180–200 kDa after reduction (fig.1, lanes 1 and 2). The 130 and 140 kDa polypeptides were obtained in a molar ratio of 1.06:1 ($\pm$ 0.08, n = 10) as demonstrated by densitometric scans of the Coomassie blue stained gel. All the bands of 130, 140 and 180–200 kDa could be stained with the PAS reagent, indicating that this fraction contains most of the glycoproteins noted above in the crude extract. In addition to these glycoproteins, our preparations contained varying amounts of a polypeptide of 45 kDa which was not PAS positive. After reduction and alkylation, the 130 and 140 kDa components could be separated from the 45 kDa and the 180–200 kDa polypeptides on a column of Sephacryl S-400 (fig.1, lane 3). Again the two bands were obtained in a molar ratio of 1:1 and were PAS positive. It is conceivable that the two components represent two genetically distinct polypeptides or that they are simply degradation or modification products of each other. To decide between these possibilities, we produced two-dimensional peptide maps of the bands excised from polyacrylamide gels (fig.2). With V8 protease, the 140 kDa band was split into four major fragments of 45–93 kDa, whereas 6 fragments of different electrophoretic mobilities were generated from the 130 kDa band. Additional peptide maps were also obtained with papain and subtilisin, these peptide maps, however, turned out more diffuse than the one obtained with V8 protease. In general, the 130 kDa band appeared to be more susceptible to enzymatic digestion than the 140 kDa band. It is therefore likely that the two bands represent distinct gene products.

In order to study the relationship between the polypeptides of 130, 140 and 180–200 kDa and type VI collagen, we prepared pepsin-extracted type VI collagen from chicken gizzard. On a polyacrylamide gel the purified protein revealed three major bands of 35, 45 and 55 kDa (termed α1-pep, α2-pep and α3-pep; fig.3) as had been

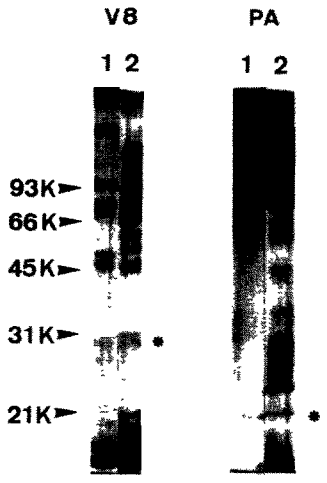

Fig.2. Peptide mapping of the 140 kDa and 130 kDa subunits. The 140 kDa chain (lane 1) and the 130 kDa chain (lane 2) were excised from a 5–10% polyacrylamide gel and digested with V8 protease (V8) or papain (PA). The resulting peptides were separated on a 10–15% polyacrylamide gel and detected by silver staining. The migration positions of globular protein standards are indicated in the left margin. Asterisks denote the positions of contaminating components present in the protease preparation.

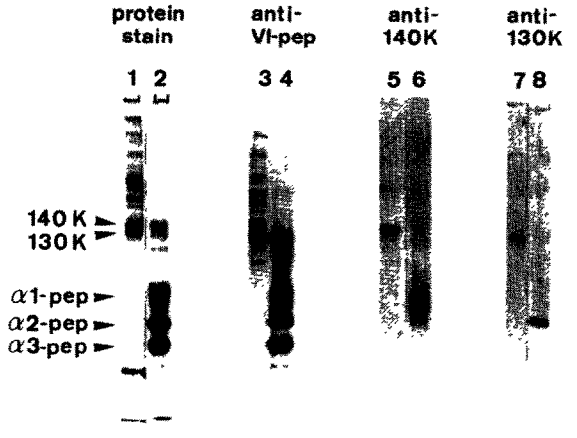

Fig.3. Identification of the individual subunits of type VI collagen by immunoblotting. Purified type VI collagen (lane 1), the crude SDS extract (lanes 3,5,7) and pepsin-extracted type VI collagen (lanes 2,4,6,8) were resolved under reducing conditions on a 5–10% polyacrylamide gel. Lanes 3–8 were transferred to nitrocellulose and incubated with affinity-purified antibodies against pepsin-extracted type VI collagen (anti-VI-pep), the 140 kDa subunit (anti-140 K) and the 130 kDa subunit (anti-130 K). Bound antibodies were visualized by ^{125}I-labelled protein A and autoradiography. Lanes 1 and 2 show a reference gel stained with Coomassie blue (protein stain).

described for bovine and human type VI collagen [1]. Antibodies against this pepsin-extracted type VI collagen reacted strongly with all the three bands on an immunoblot (fig.3, lane 4). When the SDS extract from chicken gizzard was blotted onto nitrocellulose and stained with the same antibodies, the bands of 130 kDa, 140 kDa and 180–200 kDa all showed a strong positive reaction. The same reaction was also noted with the polypeptides purified by gel filtration. Our glycoprotein preparation (fig.1, lane 2) must therefore consist predominantly of intact type VI collagen.

For further immunoblotting experiments we raised antisera against the 140 and 130 kDa bands excised from preparative polyacrylamide gels. After affinity purification, the two antibody preparations reacted strongly with their respective antigen but showed no cross-reactivity with the other polypeptide (fig.3, lanes 5–8). When pepsin-extracted type VI collagen was used for such immunoblots, antibodies against the 140 kDa band stained only the α1(VI)-pepsin fragment. On the other hand, antibodies against the 130 kDa band bound only to the α2(VI)-pepsin fragment.

Thus, the 140 kDa and the 130 kDa polypeptides, obtained in equimolar amounts, must represent the intact α1(VI) and α2(VI) subunits. Accordingly, the 180–200 kDa polypeptides may represent different modification products of the α3(VI) subunit.

4. DISCUSSION

We describe here the isolation of intact type VI collagen from a non-mammalian species. Identification of the chicken protein is based on its sensitivity towards bacterial collagenase, its carbohydrate content and its cross-reactivity with affinity purified antibodies against the pepsin-extracted collagen. The protein consists of subunits with molecular masses of 130, 140 and 180–200 kDa. Immunoblotting experiments allow the assignment of the 130 kDa subunit as the α2(VI) and of the 140 kDa subunit as the α1(VI) chain. The two chains are obtained in an equimolar ratio. Since a ratio close to 1 : 2 or an additional band in the 140 kDa region has never been observed, our results clearly disprove the hypothesis that type VI collagen is composed of three different 140 kDa subunits [4,7–9]. Our results rather support the previous observation that a much larger chain is associated with type VI collagen [10,17]. In those experiments we identified a 200 kDa component from a guanidinium extract of bovine uterus, which proved to be related to the α3(VI) subunit. In support of that work, we demonstrate now that larger chains are also found with chicken type VI collagen and show that these chains react readily with polyclonal antibodies directed against the pepsin-extracted collagen. It is therefore likely that they represent different modification or processing products of the third subunit. Based on our results, we believe that chicken type VI collagen is a disulfide-linked heteropolymer consisting of α1(VI) (140 kDa), α2(VI) (130 kDa) and α3(VI) (180–200 kDa) chains present in equimolar amounts.

To our knowledge the 140/130 kDa components of type VI collagen have never been separated into distinct polypeptide chains starting with human or bovine tissue. With cultures from human fibroblasts, however, two laboratories have noticed a closely spaced doublet [20,21] rather than a broad band in the 140 kDa region. Small species differences in the amino acid sequence may be responsible for the fact that type VI collagen from chicken tissue can readily be resolved into three subunits on a polyacrylamide gel. In spite of strenuous efforts we have unfortunately not been successful in the purification of preparative amounts of the 130 and 140 kDa polypeptide chains. Experiments with CM-cellulose, DEAE-cellulose and even reversed-phase HPLC lead to the conclusion that the two chains possess extremely similar properties in charge and hydrophobicity. For that reason, amino acid analyses and digestion experiments with bacterial collagenase have not been possible with the purified chains. Nevertheless, limited biochemical and immunological studies can be performed with the chicken subunits isolated from preparative polyacrylamide gels as demonstrated in figs 2 and 3. This may prove to be useful when the components of type VI collagen from normal cells are compared with those from malignant cells which exhibit a marked reduction in the biosynthesis of this collagen [22].

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REFERENCES

[1] Engel, J., Furthmayr, H., Odermatt, E., Von der Mark, H., Aumailley, M., Fleischmajer, R. and Timpl, R. (1985) Ann. NY Acad. Sci. 460, 25–37.
[2] Jander, R., Rauterberg, J. and Glanville, R.W. (1983) Eur. J. Biochem. 133, 39–46.
[3] Jander, R., Troyer, D. and Rauterberg, J. (1984) Biochemistry 23, 3675–3681.
[4] Von der Mark, H., Aumailley, M., Wick, G., Fleischmajer, R. and Timpl, R. (1984) Eur. J. Biochem. 142, 493–502.
[5] Heller-Harrison, R.A. and Carter, W.G. (1984) J. Biol. Chem. 259, 6858–6864.
[6] Hessle, H. and Engvall, E. (1984) J. Biol. Chem. 259, 3955–3961.
[7] Ayad, S., Chambers, C.A., Shuttleworth, C.A. and Grant, M.E. (1985) Biochem. J. 230, 465–474.
[8] Gibson, M.A. and Cleary, E.G. (1985) J. Biol. Chem. 260, 11149–11159.
[9] Aumailley, M., Von der Mark, H. and Timpl, R. (1985) FEBS Lett. 182, 499–502.
[10] Trüeb, B. and Bornstein, P. (1984) J. Biol. Chem. 259, 8597–8604.
[11] Friedman, M., Krull, L.H. and Cavins, J.F. (1970) J. Biol. Chem. 245, 3868–3871.
[12] Odermatt, E., Risteli, J., Van Delden, V. and Timpl, R. (1983) Biochem. J. 211, 295–302.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Merril, C.R., Dunau, M.L. and Goldman, D. (1981) Anal. Biochem. 110, 201–207.
[15] Eckhardt, A.E., Hayes, C.E. and Goldstein, I.J. (1976) Anal. Biochem. 73, 192–197.
[16] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[17] Trüeb, B. and Winterhalter, K.H. (1986) EMBO J. 5, 2815–2819.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Gibson, M.A. and Cleary, E.G. (1982) Biochem. Biophys. Res. Commun. 105, 1288–1295.
[20] Engvall, E., Hessle, H. and Klier, G. (1986) J. Cell. Biol. 102, 703–710.
[21] Carter, W.G. (1984) J. Cell Biol. 99, 105–114.
[22] Carter, W.G. (1982) J. Biol. Chem. 257, 13805–13815.